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Characterization of Two Unusual Guanylyl Cyclases from *Dictyostelium**

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Guanylyl cyclase A (GCA) and soluble guanylyl cyclase (sGC) encode GCs in *Dictyostelium* and have a topology similar to 12-transmembrane and soluble adenylyl cyclase, respectively. We demonstrate that all detectable GC activity is lost in a cell line in which both genes have been inactivated. Cell lines with one gene inactivated were used to characterize the other guanylyl cyclase (*i.e.* GCA in *sgc*$^-$ null cells and sGC in *gca*$^-$ null cells). Despite the different topologies, the enzymes have many properties in common. *In vivo*, extracellular cAMP activates both enzymes via a G-protein-coupled receptor. *In vitro*, both enzymes are activated by GTPγS (K_a = 11 and 8 μM for GCA and sGC, respectively). The addition of GTPγS leads to a 1.5-fold increase of V_{max} and a 3.5-fold increase of the affinity for GTP. Ca^{2+} inhibits both GCA and sGC with K_i of about 50 and 200 nM, respectively. Other biochemical properties are very different; GCA is expressed mainly during growth and multicellular development, whereas sGC is expressed mainly during cell aggregation. Folic acid and cAMP activate GCA maximally about 2.5-fold, whereas sGC is activated about 8-fold. Osmotic stress strongly stimulates sGC but has no effect on GCA activity. Finally, GCA is exclusively membrane-bound and is active mainly with Mg^{2+}, whereas sGC is predominantly soluble and more active with Mn^{2+}.

cAMP and cGMP are important signaling molecules. In prokaryotes, cAMP regulates gene expression. Cyanobacteria contain high levels of cGMP relative to other bacteria, but their function as intracellular signaling molecules is not well understood (1). In eukaryotes, cAMP and cGMP regulate enzyme activities, channel activity, and gene expression, mainly via cAMP- and cGMP-dependent protein kinase (2, 3). A large and complex family of adenylyl cyclase (AC)[1] and guanylyl cyclase (GC) is responsible for the synthesis of cAMP and cGMP (4, 5).

The crystal structure of mammalian AC (6, 7) suggests that the core of the enzyme consist of two cyclase domains that are associated in an antiparallel manner. In metazoan, four cyclase subgroups are recognized (4, 5, 8): 1) the 12-transmembrane adenylyl cyclase is composed of two different cyclase domains and is regulated by G-proteins; 2) The single-transmembrane guanylyl cyclase contains one cyclase domain and functions as a homodimer, and GC activity is stimulated by extracellular peptides; 3) The nitric oxide-sensitive soluble guanylyl cyclase is a complex of two different proteins with one cyclase domain each; 4) The recently discovered soluble adenylyl cyclase (sAC) from rat and human possesses two cyclase domains, which share the highest degree of identity with bacterial adenylyl cyclases (9).

In the social amoeba *Dictyostelium*, cGMP is implicated as one of the second messengers for chemotaxis (10), although its precise role is not known. On the other hand, cAMP can act as both first and second messenger (11, 12). As first messenger, cAMP induces chemotaxis, cAMP signal relay, and gene expression. *Dictyostelium* possesses three AC and two GC genes. ACA is similar to mammalian 12-transmembrane AC and controls cAMP signaling during cell aggregation. ACG is an AC with the topology of a membrane-bound GC containing one cyclase domain and one transmembrane region (13); the enzyme produces cAMP, which regulates spore germination. ACB, encoded by the *acrA* gene, is most similar to cyanobacterial AC and controls spore maturation (14–16). Recently we identified two *Dictyostelium* GC gene, *gcaA* and *sgcA*, encoding GCA (17) and sGC (18), respectively. The deduced amino acid sequences and predicted topologies suggest that GCA and sGC are more related to animal AC than to animal GC enzymes. GCA has the topology of 12-transmembrane AC in which the two cyclase domains appear to be functionally swapped. Thus, the second cyclase domain of mammalian AC, which provides most catalytic interactions with ATP, is similar to the first domain of GCA interacting with GTP. GC enzymes with this topology have also been found in *Paramecium* and *Plasmodium* (19, 20), but unlike GCA these proteins have a P-type ATPase at their N terminus. The second *Dictyostelium* guanylyl cyclase, sGC, contains two cyclase domains and two long (~1000 amino acids) N- and C-terminal regions. The cyclase domains and the C-terminal region of sGC shows a high degree of identity with the corresponding segments of human soluble AC. Interestingly, homologs of sAC are present in bacteria and rat but are absent from the completely sequenced genomes of *Drosophila*, *Caenorhabditis elegans*, *Arabidopsis*, and yeast. Phylogenetic analysis places the *Dictyostelium* sGC as the evolutionary intermediate between the bacterial and vertebrate sequences (18, 21).

To better understand the role of cGMP and GC enzymes in *Dictyostelium*, we have characterized GCA and sGC. First we show that all GC activity is lost in a cell line in which both genes are inactivated, suggesting that GCA and sGC represent all GC activity in *Dictyostelium*. Subsequently, GCA was characterized in a cell line with a deletion of sGC, and sGC was characterized in a strain without GCA.

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[1] The abbreviations used are: AC, adenylyl cyclase; GC, guanylyl cyclase; s, soluble; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate.

MATERIALS AND METHODS

Strains and Culture Conditions—AX3 (wild type), *gca*$^-$ null cells (17), *sgc*$^-$ null cells (18), and *gca*$^-$/*sgc*$^-$ double null cells (see below) were grown in HG5 medium. When grown with selection, HG5 medium was supplemented with 10 μg/ml blasticidine S. To select for complementation of the uracil auxotroph DH1 cells by the pyr5/6 cassette (see below), cells were grown in uracil-deficient FM medium (22) (ICN). Cells were starved for up to 6 h by shaking in 10 mM phosphate buffer, pH 6.5, at a density of 10^7 cells/ml.

Gene Inactivation—The *gca*$^-$/*sgc*$^-$ double knock-out cell lines were obtained in the uracil auxotroph strain DH1 using the *pyr5/6* gene (23) and the *bsr* gene (24) as selection markers. The *pyr5/6* cassette contains the coding region of the Pyr5/6 protein (23), which was amplified by PCR and cloned into a vector between the actin-15 promoter and the actin-8 terminator. To make the double knock-out strain, first sGC was inactivated in DH1 using the knock-out construct of sGC (see Ref. 18) in which the DNA segment encoding the Bsr selection marker was replaced by the pyr5/6 selection cassette, yielding psCycKOpyr. A linear fragment with the sGC flanks and the pyr5/6 selection cassette was obtained by two rounds of PCR. Homologous integration of this fragment in DH1 resulted in the replacement of the region coding for amino acids 1192–1224 of sGC for the pyr5/6 selection cassette. Subsequently, two independent *sgc* knock-out clones were used to disrupt the GCA gene with the Bsr-containing construct as described previously (17). The disruption of both genes was confirmed by PCR as well as by Northern analysis. Two independent clones were used in this study.

Guanylyl Cyclase Assays—AX3, *gca*$^-$ null, *sgc*$^-$ null, and *gca*$^-$/*sgc*$^-$ null cells were harvested and starved for 1 or 5 h in phosphate buffer. Unless mentioned otherwise, the procedure for determining GC activity was as follows. Cells were washed and resuspended in ice-cold lysis buffer (1.5 mM EGTA and 250 mM sucrose in 10 mM Tris, pH 8.0) and lysed through nucleopore filters (pore size, 3 μm) in the absence or presence of 100 μM GTPγS. For separation into soluble and particulate fractions, 0.5-ml aliquots of filter lysates were centrifuged for 1.5 min at 14,000 $\times$ *g*, and the pellets were resuspended in 0.5 ml of lysis buffer (particulate fraction). Lysates and fractions were incubated at 22 °C with 0.5 mM GTP in the presence of 10 mM dithiothreitol, 0.2 mM 3-isobutyl-1-methylxanthine, and 2 mM Mn^{2+} or Mg^{2+} in 0.75 mM EGTA, 250 mM sucrose, and 15 mM Tris, pH 8.0. Reactions were terminated after 30 and 60 s by the addition of an equal volume of 3.5% perchloric acid. After neutralization, cGMP levels were measured by radioimmunoassay (25). The data shown are generally the means and standard error of the mean from three independent experiments each with lysis in triplicates.

cGMP Response to Folic Acid, cAMP, and Osmotic Stress—Cells were starved for 1 h to measure the cGMP response to folic acid or osmotic stress and for 5 h to measure the response to cAMP. Subsequently, cells were washed, resuspended in phosphate buffer at 10^8 cells/ml, and stimulated with 1 μM folic acid, 300 mM glucose, or 0.1 μM cAMP. The reactions were terminated with perchloric acid, and cGMP levels were measured by radioimmunoassay.

RESULTS

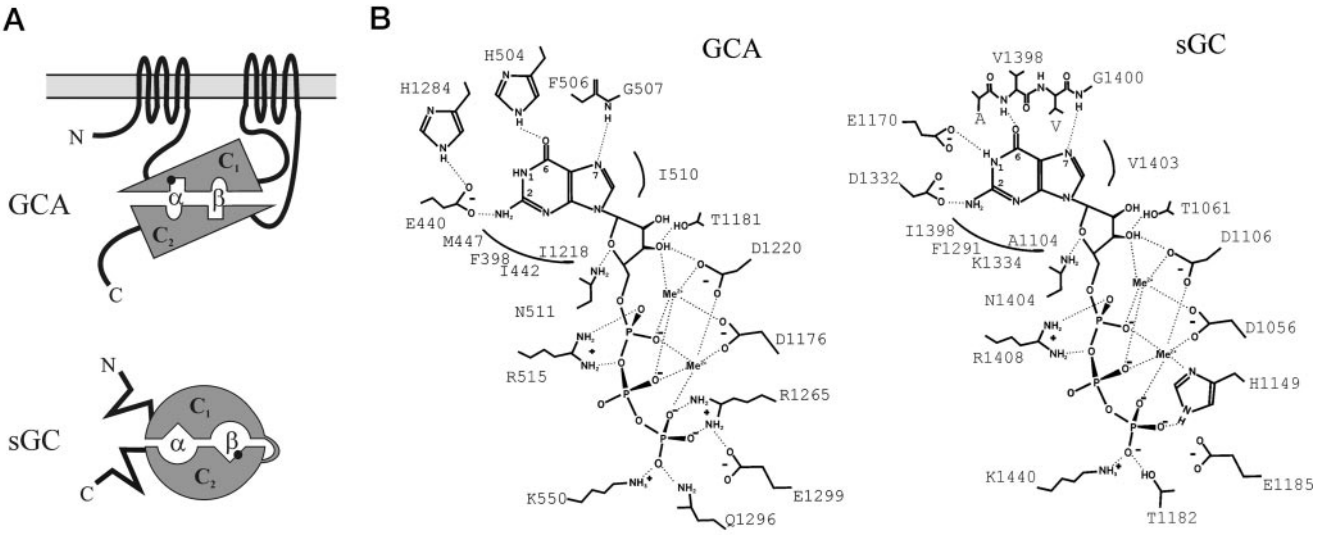
Topology and Catalytic Pockets of GCA and sGC—*Dictyostelium* contains two genes that encode guanylyl cyclase enzymes, GCA and sGC. Both enzymes contain two cyclase domains, which is essentially the only feature that these enzymes have in common (Fig. 1*A*). GCA has the topology of a membrane-bound mammalian adenylyl cyclase with two stretches of six transmembrane-spanning segments. In contrast, sGC has the topology of soluble mammalian adenylyl cyclase, including a homologous long C-terminal segment. Based on the three-dimensional structure of mammalian adenylyl cyclase, the two cyclase domains are expected to form an antiparallel dimer with potentially two catalytic pockets, α and β. In mammalian adenylyl cyclase, ATP is bound in the β pocket; the α pocket contains many amino acids that would prevent binding and catalysis of ATP (6, 7). Instrumental in this respect is the transition state stabilizing Arg^{1029} from the C2 domain of mammalian adenylyl cyclase. The amino acids that cover the potential binding pockets of sGC indicate that GTP is bound in the β pocket of sGC (Fig. 1, *B* and *C*). In contrast, in GCA the catalytic arginine is provided by the C1 domain, and consequently GTP is predicted to be bound and hydrolyzed in the α pocket.

The three-dimensional structure of mammalian adenylyl cyclase with bound ATP analogs in the catalytic site suggests that two aspartates (Asp^{396} and Asp^{440}) interact via two metal ions with the triphosphate moiety of ATP (35). These two metal-binding aspartates are conserved in GCA and sGC (Fig. 1, *B* and *C*). The γ-phosphate of ATP interacts with two positively charged amino acids (Arg^{484} and Lys^{1065} in AC), of which one (Arg^{484}) forms a salt bridge to Glu^{518}. These amino acids are conserved in GCA and sGC as well, except for Lys^{1065}, which is a histidine in sGC (His^{1149}). Although this histidine may still interact with Pγ, it can no longer interact with the conserved glutamate (Glu^{1185}) unless it is protonated.

The purine moiety is bound to AC in a cleft that contains many hydrophobic amino acids. These hydrophobic amino acids are conserved in GCA and sGC, except for a lysine in sGC (Lys^{1334}). Interestingly, sAC, the mammalian homolog of sGC, also contains a lysine at this position. The substrate specificity of cyclases is determined predominantly by a lysine for adenylyl cyclases (Lys^{938} in ACII), which is a glutamate in guanylyl cyclases (Glu^{928} in GCE). Both GCA and sGC comply with this general observation, as they do not possess the positively charged lysine but contain the negatively charged glutamate in GCA and aspartate in sGC. In mammalian GCE it has been modeled that the O-6 of the guanidine moiety forms a weak hydrogen bond to the side chain thiol of Cys^{1000}. This hydrogen bond may still be possible with the histidine at this position in GCA (His^{504}) but not with the alanine in sGC (Ala^{1397}); perhaps in sGC the O-6 group forms a hydrogen bond with the backbone amide of Val^{1398}. Glu^{928} of GCE has been modeled to interact with Arg^{998}. At the position of this arginine GCA contains a histidine (His^{1284}) that may fulfill this function. In contrast, in sGC this amino acid is replaced by a glutamate (Glu^{1170}), which cannot provide the interaction with the corresponding aspartate; perhaps Glu^{1170} interacts with the N1H of guanine in sGC.

The noncatalytic pocket of GCA as well as that of sGC contain many hydrophobic amino acids and lack the amino acids that bind the metal ion and the phosphates of GTP. In this respect they have the properties of the noncatalytic sites of many mammalian adenylyl and soluble guanylyl cyclases. Forskolin, which binds in the noncatalytic site of mammalian membrane adenylyl cyclase, does not affect GCA or sGC activity (Ref. 17 and data not shown).

Inactivation of GCA and sGC—To investigate whether GCA and sGC encode all guanylyl cyclases in *Dictyostelium*, these two genes were inactivated separately and in combination. Wild-type cells contain high levels of Mg^{2+}-dependent guanylyl cyclase activity (Fig. 2). Guanylyl cyclase activity in *sgc*$^-$ cells is reduced to about 30% of the activity of wild-type cells. In *gca*$^-$ cells, the reduction of guanylyl cyclase activity is much smaller, to about 65% of that in wild-type cells. The double null mutant *sgc*$^-$/*gca*$^-$ does not contain detectable guanylyl cyclase activity either with Mg^{2+}/GTP (Fig. 2) or with Mn^{2+}/GTP as substrate (data not shown). The sensitivity of the assay implies that the guanylyl cyclase activity in the double null cells is maximally 1% of the activity in wild-type cells. Basal cGMP levels are significantly reduced in *sgc*$^-$ cells and slightly reduced in *gca*$^-$ cells. Again, the double null cell line does not contain cGMP above the detection limit (Fig. 2). Additional experiments at different developmental stages and measurements of intracellular cGMP levels *in vivo* after cAMP stimulation all failed to detect significant levels of guanylyl cyclase or cGMP in the double null cells (data not shown). The guanylyl cyclase double null cells can aggregate and form fruiting bodies; these cells show reduced chemotaxis with approximately the same mild defects as *sgc*$^-$ cells (data not shown).



C

Amino acid in ACV/ACII	Function	Catalytic sites: Adenylyl cyclases Rn AC V/II β	Hs sAC β	Guanylyl cyclases Rn GC sol β	Rn GCE αβ	Par GC α	*Dictyostelium* Guanylyl cyclases: Dd sGC α	β	Dd GCA α	β	Non-catalytic site: Adenylyl cyclases Rn AC V/II α	Hs sAC α	Guanylyl cyclases Rn GC sol α	Par GC β
Purine	Recognition													
K938 C2	K in AC, E in GC	K	***F***	E	E	E	K	D1332	E440	A	K	K	E	Y
D1018 C2	D in AC, C in GC	D	***T***	C	C	S	L	***A1397***	***H504***	Q	D	L	C	D
Q582 C1	Q in AC, R in GC	Q	***H***	R	R	R	E	***E1170***	***H1284***	S	Q	E	R	Q
V1024 C2	Hydrophobic pocket	V	V	V	V	I	V	V1403	I510	V	V	L	V	V
M945 C2		M	L	M	M	M	L	I1398	M447	F	Y	L	C	V
F899 C2		F	F	F	F	F	F	F1291	F398	L	F	F	F	F
I940 C2		I	***K***	V	I	V	A	***K1334***	I442	I	L	A	I	I
L438 C1		L	A	I	I	I	K	A1104	I1218	I	I	K	V	V
Catalytic	Essential													
N1025 C2		N	N	N	N	N	I	N1404	N511	Q	T	D	T	L
R1029 C2		R	R	R	R	R	L	R1408	R515	Q	H	L	K	K
Me^{2+}	Essential													
D440 C1		D	D	D	D	D	C	D1106	D1220	N	S	C	D	K
D396 C1		D	D	D	D	D	N	D1056	D1176	E	S	N	G	D
Ribo-Phosph.														
T401 C1	3'OH	T	T	T	T	T	T	T1061	T1181	N	K	D	N	D
R484 C1	Pγ	R	K	R	R	K	S	***H1149***	R1265	R	R	S	R	K
E518 C1	Salt bridge to R484	E	Q	E	E	E	M	E1185	E1299	E	D	M	E	C
K1065 C2	Pγ	K	K	K	K	K	E	K1440	K550	I	Y	F	F	I
N515 C1	Pγ (STNOKR)	N	R	S	N	N	A	T1182	Q1296	S	S	A	N	S

FIG. 1. **Two unusual guanylyl cyclases, *Dictyostelium* GCA and sGC.** *A*, schematic of the topology of GCA and sGC with the two cyclase domains associated as an antiparallel dimer. The catalytic site is located at the interface of the two domains but not in the center. Therefore, there are two potential catalytic pockets, α and β. In a homodimer, such as in membrane-bound mammalian guanylyl cyclase GCE, the α and β catalytic sites are identical. However, in heterodimers α and β could be different. The catalytic arginine (Arg^{1029} in ACII) is indicated by a *dot*. Sequence alignment indicates that GTP is hydrolyzed in the α-site of GCA and in the β-site in sGC (as in all vertebrate cyclases). *B*, model of the interaction of GTP with amino acid side chains of GCA and sGC based on the three-dimensional structure of ACII/V (6, 35) and modeling of GCE (46). *C*, the table with the main interactions of substrates with amino acids of membrane-bound and soluble AC (*ACV/II* and *sAC*), membrane-bound and soluble GC (*GCE* and *GCsol*), *Paramecium* GC (*GCpar*), and the *Dictyostelium* guanylyl cyclases, GCA and sGC. The data are based on sequence alignment and the identification of the amino acids that interact with ATP in the crystal structure of ACII/V. The active catalytic site is defined as the α or β pocket that contains the essential catalytic amino acids (Asn^{1025} and Arg^{1029} in ACII/V). In GCE, which is a homodimer, both the α- and β-sites are identical and catalytically active. Unusual amino acids are indicated in *boldface italics*.

The results indicate that GCA and sGC account for all guanylyl cyclase activity in *Dictyostelium*. Consequently, this implies that sgc^- cells can be used to characterize GCA without background of any other guanylyl cyclase, and conversely, gca^- cells can be used to characterize sGC.

Mg^{2+} and Mn^{2+} Dependence of GCA, and sGC and Inhibition by Ca^{2+}—Adenylyl and guanylyl cyclases require bivalent cations to support enzyme activity. In Fig. 3 dose-response curves are shown for gca^- and sgc^- cells at different concentrations of Mg^{2+} and Mn^{2+}. The activity of sGC (*gca-* cells) is about 5-fold higher with Mn^{2+}/GTP than with Mg^{2+}/GTP, with maximal activity at 1–2 mM for both cations (Fig. 3*A*). Surprisingly, GCA (sgc^- cells) is predominantly active with Mg^{2+}/GTP, whereas Mn^{2+}/GTP support only about 30% of the Mg^{2+}-dependent activity (Fig. 3*B*). As the intracellular concentration of Mn^{2+} is only 10 μM and Mg^{2+} reaches a concentration of 3 mM (26), the

physiological substrate of both enzymes is probably Mg^{2+}/GTP.

Ca^{2+} ions are known to inhibit Mg^{2+}-dependent guanylyl cyclase in *Dictyostelium* (27–29). Fig. 4 reveals that both GCA and sGC are sensitive to Ca^{2+} inhibition. The Ca^{2+} dose dependence suggests that GCA is slightly more sensitive to Ca^{2+} inhibition than sGC, showing half-maximal inhibition at about 50 nM for GCA and at about 200 nM for sGC. The Mn^{2+}-dependent activity of sGC (18) or GCA (data not shown) is not inhibited by 10 μM Ca^{2+}.

Kinetics of GCA and sGC—cGMP formation is activated *in vivo* by extracellular cAMP via a G-protein-coupled signal transduction pathway. Expression of the G-protein subunits Gα2 and the single Gβ are essential to activate guanylyl cyclase (12). The Mg^{2+}-dependent guanylyl cyclase activity is stimulated *in vitro* by GTPγS (10). We observed that GTPγS has no effect on Mn^{2+}-dependent sGC (18) or GCA activity (data not shown). To investigate how GTPγS regulates guanylyl cyclase activity, we determined the K_m and V_{max} of GCA and sGC for Mg^{2+}/GTP in the absence and presence of GTPγS (Fig. 5). The results indicate that both enzymes show Michaelis-Menten kinetics without indications for cooperativity and that GTPγS stimulates enzyme activities by increasing the V_{max} and reducing the K_m of both enzymes. The K_m of GCA for GTP is 250 ± 50 μM (Fig. 5*A*). GTPγS activates GCA by reducing the K_m to 66 ± 2 μM GTP. In addition, it induces a moderate increase of the V_{max} from 6.9 ± 0.7 to 9.8 ± 0.1 pmol/min/mg protein. The effect of GTPγS on sGC is approximately the same (Fig. 5*B*); it reduces the K_m from 414 ± 110 to 112 ± 10 μM and increases the V_{max} from 16 ± 3 to 27 ± 1 pmol/min/mg protein. The GTPγS dose dependence of enzyme activation is presented in Fig. 5*C*. The curves are best fitted by an equation with a Hill coefficient of 1.7 ± 0.4 for both GCA and sGC. Half-maximal activation of GCA is induced by 11 ± 2 μM GTPγS and by 8 ± 2 μM GTPγS for sGC. The data suggest that GCA and sGC are regulated by GTPγS via a similar mechanism.

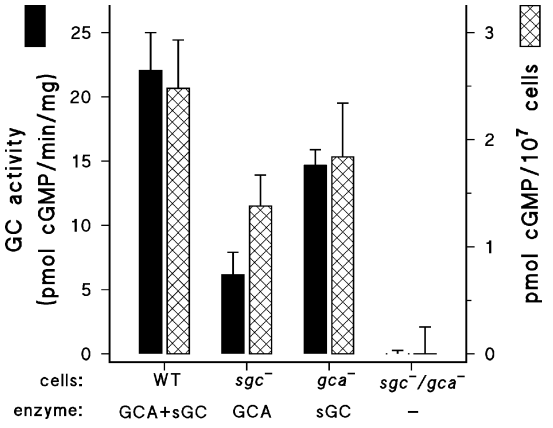


FIG. 2. **GC activity and basal cGMP levels in strains with a disruption of the genes encoding GCA or sGC.** Cells were starved for 1 h and lysed either in perchloric acid for the determination of basal cGMP levels (*hatched bars*) or in the presence of GTPγS for the determination of Mg^{2+}-dependent GC activity (*filled bars*). The strains used are wild-type cells (*WT*), cells with a deletion of GCA (*gca*$^-$) or a deletion of sGC (*sgc*$^-$), or cells with a deletion of both GCA and sGC (*gca*$^-$/*sgc*$^-$). The GC enzymes that are present in these cells are indicated *below* the names of the strains.

Developmental Expression of GCA and sGC—Northern blots have suggested that GCA is expressed in wild-type cells mainly during growth and development, whereas sGC is expressed maximally during cell aggregation (17, 18). For quantitation of these Northern blots (Fig. 6*A*) the expression levels for GCA and sGC during development were normalized for the expression of each gene at 0 h of development. The expression of GCA shows a 70% decline at the onset of cell aggregation (4 h), whereas the expression of sGC increases about 2-fold during cell aggregation. In the multicellular stage, the expression of GCA increases strongly to reach a maximum in the slug stage, which is about 2-fold higher than during growth and 10-fold higher than during aggregation. The expression levels of sGC in the multicellular stages decline to the levels during growth.

GCA activity in vegetative *sgc*$^-$ cells is about 6 pmol/min/mg protein, which decreases significantly to 2.5 pmol/min/mg protein in starved cells (Fig. 6*B*). The Mg^{2+}-dependent activity of sGC in vegetative *gca*$^-$ cells amounts to 11 pmol/min/mg proteins and increases to about 17 pmol/min/mg protein during aggregation. These data indicate that during growth 20–40% of the Mg^{2+}-dependent GC activity is attributed to GCA and 60–80% to sGC. After starvation the total activity increases about 1.5-fold; ~90% is attributable to SGC and ~10% to GCA. The reduction in GCA and increase in sGC activity are consistent with the developmental changes of mRNA expression seen on Northern blots.

Stimulation of GCA and sGC by cAMP, Folic Acid, and Osmotic Stress—Folic acid, cAMP, and osmotic stress induce

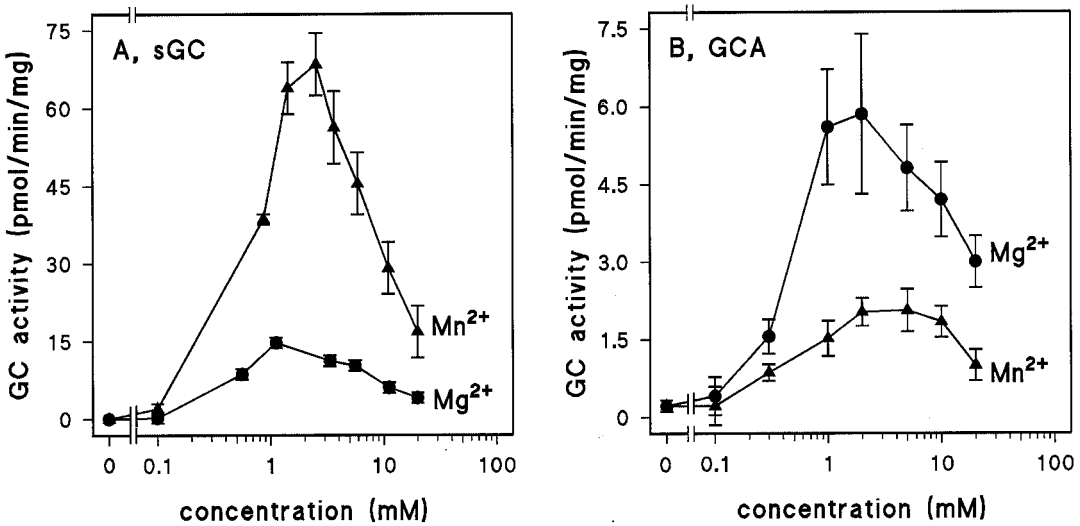


FIG. 3. **Mn^{2+} and Mg^{2+} dependence of GCA and sGC activity.** Cells were starved and lysed, and GC activity was measured with 0.5 mM GTP and different concentration of Mn^{2+} (235) or Mg^{2+} (234). Mg^{2+}-dependent activity was measured in the presence of GTPγS. The abscissa indicates the free Mg^{2+} and Mn^{2+} concentrations, the total concentrations from which the concentrations of GTP (0.5 mM), GTPγS (50 μM) and EGTA (0.75 mM, for Mn^{2+} only) were subtracted. *A*, 5-h starved *gca*$^-$ cells for sGC. *B*, 1-h starved *sgc*$^-$ cells for characterization of GCA.

the activation of guanylyl cyclase activity in wild-type cells (10). These responses were analyzed in *gca*$^-$ and *sgc*$^-$ null cells to investigate which of the two guanylyl cyclases is activated by these agents (Fig. 7). In *sgc*$^-$ cells (regulation of GCA), the responses are small (folic acid and cAMP) or absent (osmotic stress), indicating that GCA is not or is weakly activated by these agents. In contrast, in *gca*$^-$ cells (sGC regulation), guanylyl cyclase activity is potently activated by folic acid, cAMP, and osmotic stress, indicating that sGC is strongly stimulated by these compounds.

Quantitation of these responses suggests that GCA contributes about 55% to the basal cGMP levels of vegetative wild-type cells and about 40% in starved cells. Folic acid stimulation of *sgc*$^-$ cells leads to a 2.2-fold increase of GCA-produced cGMP levels (*filled bar* in Fig. 7*A*) and cAMP stimulates cGMP levels about 2.7-fold in these cells (Fig. 7*B*). Interestingly, GCA is insensitive to osmotic stress, as *sgc*$^-$ cells show no increase of cGMP levels upon stimulation with 0.3 M sucrose (Fig. 7*C*). In contrast to the weak responses of GCA, sGC is potently stimulated by these agents, because in *gca*$^-$ cells folic acid and cAMP induce a 5.5- and 8-fold increase, respectively, in cGMP levels. The increase of cGMP levels after stimulation of wild-type cells by osmotic stress is totally attributable to sGC, because this response is fully preserved in *gca*$^-$ cells and is absent in *sgc*$^-$ cells.

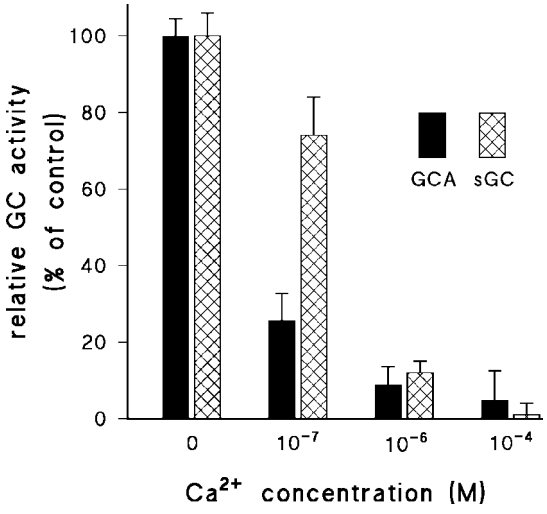


FIG. 4. **Inhibition of GCA and sGC by Ca^{2+} ions.** Mg^{2+}-dependent GC activity was measured in *sgc*$^-$ cells for GCA (*solid bars*) and in *gca*$^-$ cells for sGC (*hatched bars*). Ca^{2+}/EGTA buffers were used to obtain the indicated free Ca^{2+} concentrations. The incubation without added Ca^{2+} is indicated as *0*; the amount of cell-derived Ca^{2+} (maximally 5 μM in the assay) predicts that the free Ca^{2+} concentration will be 10^{-9} M or less.

DISCUSSION

Dictyostelium contains two genes that encode for unusual GC enzymes, GCA and sGC. Amino acid sequence alignment, phylogenetic studies, and topology suggest that sGC belongs to the small group of soluble AC enzymes present in human, rat, and some bacteria (18, 30). GCA belongs to the large group of prevailing cyclases that harbors nearly all vertebrate ACs and GCs; GCA is most closely related to the family of 12-transmembrane ACs (17). Sequence alignment of cyclase domains and site-directed mutagenesis suggest that one amino acid may be crucial in determining substrate specificity of cyclases, which is a lysine in nearly all adenylyl cyclases and a glutamate in guanylyl cyclase (6, 31–34). In GCA this amino acid is glutamate (Glu^{440}) and in sGC an aspartate (Asp^{1332}). Two other amino acids proposed to be important for determining substrate specificity are an aspartate and a glutamine in adenylyl cyclases, which are an arginine and cysteine at the same positions in guanylyl cyclases. These amino acids are not conserved in GCA and sGC, as they are replaced by two histidines in GCA and by a glutamate and alanine in sGC. Although it is possible to provide a function for these amino acids in substrate recognition (Fig. 1*B*), it would be interesting to determine the three-dimensional structure of these unusual cyclases. Mutagenesis of GCA has shown that replacing the glutamate and histidine to the corresponding lysine and aspartate converts GCA into a fully active adenylyl cyclase (34). The notion that GCA and especially sGC contain many unusual amino acids at positions that have been shown to provide substrate specificity suggests that conversion of an adenylyl to a guanylyl cyclase can be achieved in different ways.

Regulation of adenylyl and guanylyl cyclases by their natural effectors such as G-proteins or Ca^{2+} is generally detectable only with Mg^{2+}, whereas Mn^{2+} uncovers all intrinsic activity (35). This notion also holds for sGC because this enzyme is more active with Mn^{2+}/GTP than with Mg^{2+}/GTP, whereas GTPγS and Ca^{2+} strongly affect Mg^{2+}-dependent activity but have no effect in the presence of Mn^{2+}. Interestingly, GCA is active predominantly with Mg^{2+}/GTP as substrate, whereas with Mn^{2+} the activity is reduced at least 3-fold. A trivial but unprecedented explanation for the low Mn^{2+}-dependent GCA

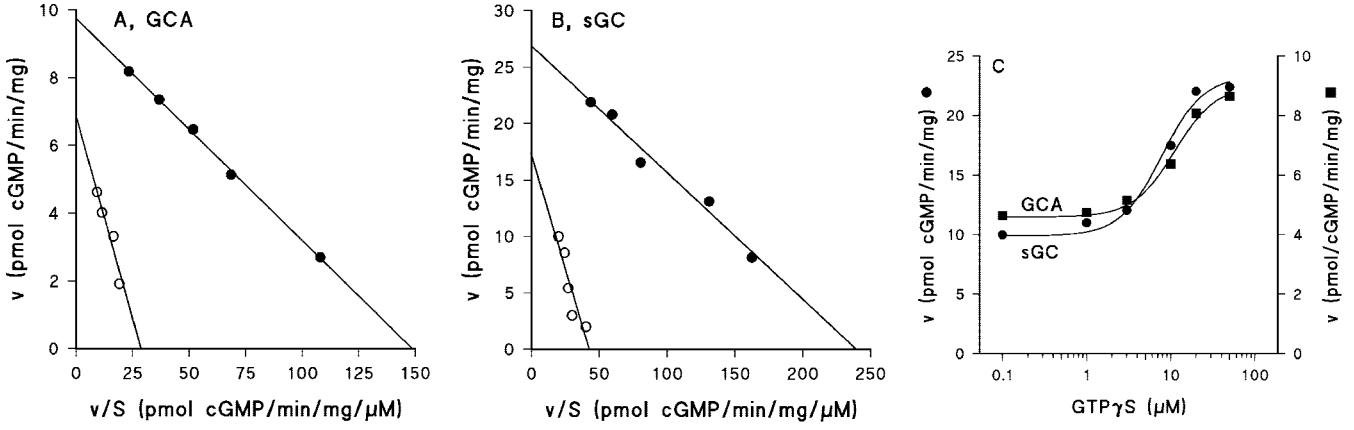


FIG. 5. **Kinetics of GCA and sGC.** *A*, Mg^{2+}-dependent GC activity was measured for GCA in *sgc*$^-$ cells at different concentrations of GTP in the presence (○) or absence (●) of 50 μM GTPγS. *B*, same as in *A* but using *gca*$^-$ cells for sGC. *C*, Mg^{2+}-dependent GC activity was measured for GCA in *sgc*$^-$ cells and for sGC in *gca*$^-$ cells at 0.5 mM GTP and different concentrations of GTPγS. The *curves* represent linear regression analysis (*A* and *B*) and a Hill equation (*C*), respectively; this allows the determination of the K_m and V_{max} for GTP of GCA (*A*) and sGC (*B*) and the K_a and Hill coefficient for GTPγS (*C*). The results suggest that both GCA and sGC are activated by GTPγS with a K_a ~10 μM and a Hill coefficient ~ 1.7; activation is due to an ~1.5-fold increase of V_{max} and an ~3.5-fold decrease of K_m.

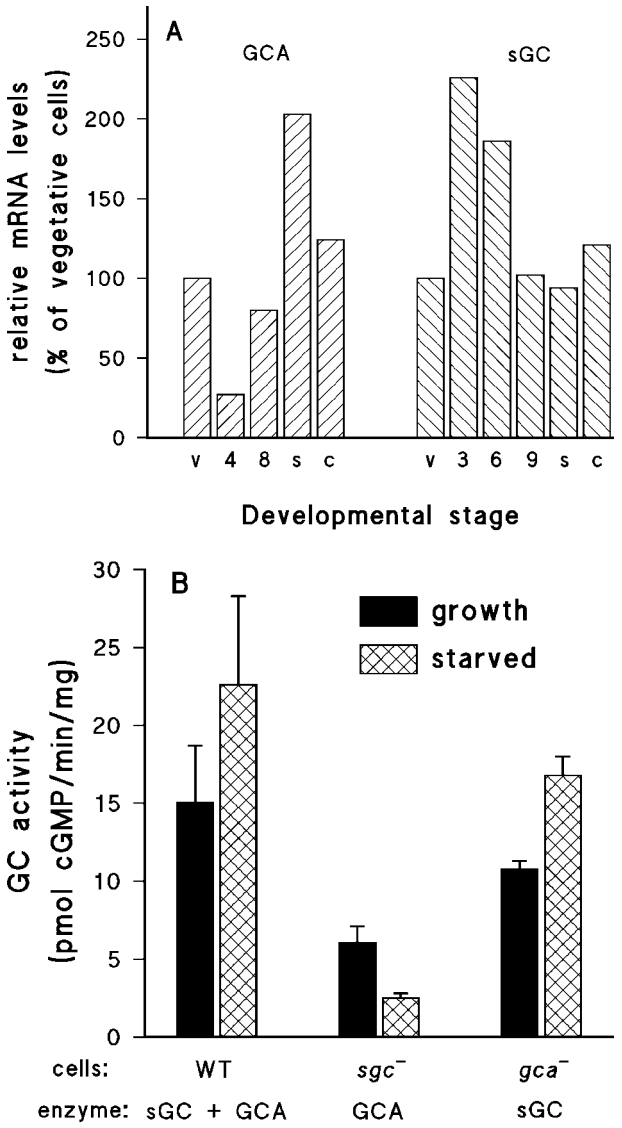


FIG. 6. **Developmental expression of GCA and sGC.** *A*, mRNA was isolated from wild-type cells that were starved for different periods. Northern blots of this mRNA were probed with DNA-encoding fragments of GCA or sGC. The data shown are a quantification of these Northern blots and are presented for each gene relative to the expression in vegetative cells. The developmental stages indicate hours of starvation (*numbers*), vegetative cells (*v*, 0 h), slugs (*s*, ~14 h), and culminants (*c*, about 22 h). *B*, Mg^{2+}-dependent GC activity was measured in lysates from 1- and 5-h starved cells (sgc^- for GCA and gca^- for sGC). Cells were lysed in the presence of GTPγS. The data suggest that GCA activity is generally lower than sGC activity and that GCA is expressed mainly during growth and late development, whereas sGC is expressed mainly during aggregation.

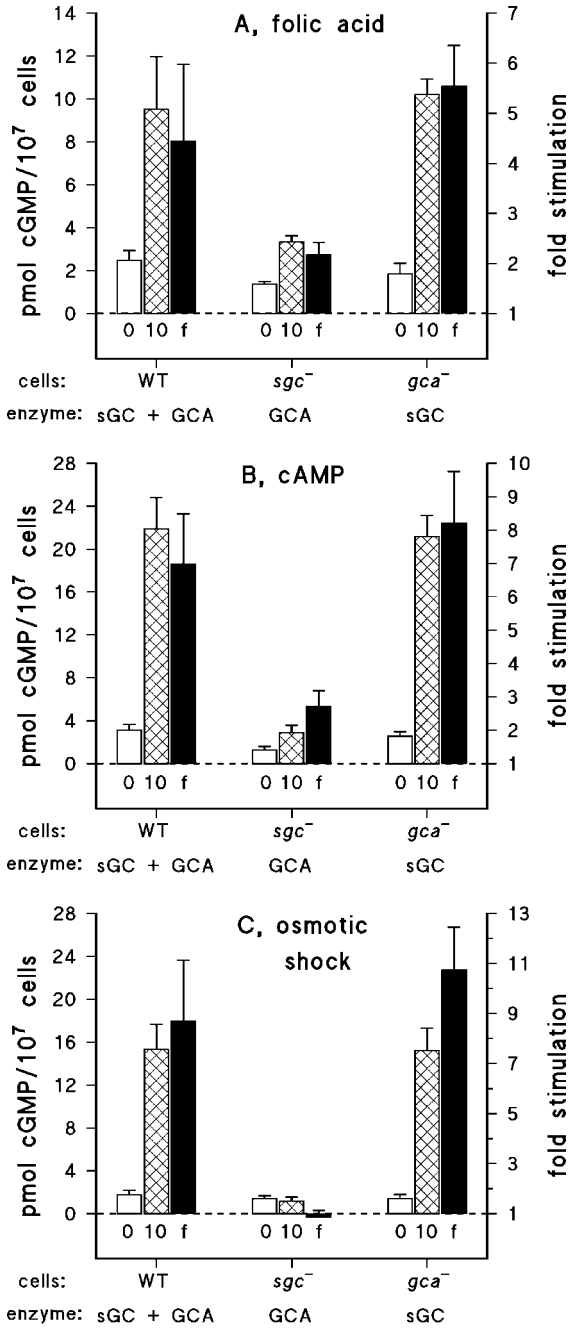


FIG. 7. ***In vivo* stimulation of GCA and sGC by folic acid, cAMP, and osmotic stress.** Cells were starved for 1 h (folic acid and osmotic stress) or 5 h (cAMP) and stimulated with 1 μM folic acid (*A*), 0.1 μM cAMP (*B*), or 300 mM glucose (*C*). Cells were killed with perchloric acid just before stimulation (*open bars*) or with folic acid and cAMP 10 s after stimulation and with glucose 10 min after stimulation (*hatched bars*). cGMP levels were determined in the neutralized lysates. The fold stimulation levels are presented as *filled bars*.

activity could be that Mn^{2+}-dependent GCA activity remains inhibited by Ca^{2+}. Although lysates contain EGTA to chelate Ca^{2+}, Ca^{2+} will be released during the GC assay because EGTA has a higher affinity for Mn^{2+} than for Ca^{2+} (36). We are not aware of a chelator that binds Ca^{2+} with 10,000-fold higher affinity than Mn^{2+}, which would be needed to test the hypothesis. However, the small Mn^{2+}-dependent GCA activity could not be inhibited by the addition of 10 μM Ca^{2+}, suggesting that GCA either has low intrinsic Mn^{2+}-dependent activity or that the low Mn^{2+}-dependent activity represents a residual Ca^{2+}-insensitive activity.

Despite the different sensitivities of GCA and sGC to bivalent cations, the physiologically relevant cation is probably Mg^{2+} for both sGC and GCA, because the intracellular concentration of Mn^{2+} (~10 μM) is too low to support activity, whereas the Mg^{2+} concentration (~3.5 mM) is sufficient (26). It is expected that GCA with 12 hydrophobic segments is found in the particulate fraction of a cell lysate and that sGC without hydrophobic segments resides in the soluble fraction. All Mg^{2+}- and Mn^{2+}-dependent GCA activity was found in the particulate fraction.[2] Previously (18) we demonstrated that a significant portion of sGC (~20% of Mn^{2+}-dependent activity) is measured in the particulate fraction and is equally active with Mg^{2+}, whereas the large soluble Mn^{2+}-dependent activity is nearly

[2] J. Roelofs and P. J. M. Van Haastert, unpublished observations.

inactive with Mg^{2+}/GTP as substrate. Thus, both GCA and sGC are membrane-associated enzymes with the physiologically relevant cation Mg^{2+}.

GCA as well as sGC can be stimulated by the chemoattractants folic acid and cAMP, although maximal stimulation of GCA is much weaker (2.5-fold) than maximal stimulation of sGC (8-fold). There is no simple explanation for this difference, because potential regulators such as GTPγS and Ca^{2+} have nearly the same effect on GCA as on sGC. Perhaps sGC is activated by additional mechanisms, a supposition that is also supported by the observation that osmotic stress activates sGC but not GCA. Translocation of soluble sGC to the membrane or modification of soluble sGC by which it becomes active with Mg^{2+}/GTP could provide the additional activation of sGC that is not possible for GCA.

The mechanism by which GTPγS stimulates GCA and sGC is not straightforward. Ample evidence indicates that *in vivo* chemoattractants stimulate cGMP formation through a G-protein-mediated pathway. No detectable cGMP formation occurs upon stimulation of cells in which the single Gβ subunit has been deleted, suggesting that chemotactic stimulation of GCA and sGC are both mediated via heterotrimeric G-proteins (37). *In vitro* GTPγS stimulates both GCA and sGC by inducing a 1.5-fold increase of the V_{max} of the enzyme and a 3.5-fold reduction of the K_m for GTP. Also the K_a and Hill coefficient for the GTPγS dose dependence of GC stimulation are similar for both enzymes, which may suggest that GCA and sGC are regulated by the same GTP-binding protein. The *in vitro* GTPγS activation of sGC and GCA could represent the aforementioned essential requirement of heterotrimeric G-proteins for chemoattractant-mediated stimulation *in vivo*. However, several anomalies have been observed that are not consistent with a heterotrimeric G-protein as the target for GTPγS-mediated GC activation. First, GTPγS still stimulates GC activity in lysates from Gβ-null cells (37), suggesting that at least the prevailing sGC is still sensitive to GTPγS. In addition, upon mutation of GCA to an adenylyl cyclase, the enzyme is still stimulated by GTPγS when expressed in cells with a deletion of Gβ or two Gα subunits (34). Second, both GCA and sGC show normal Michaelis-Menten kinetics, suggesting that GTP does not stimulate these enzymes. Third, it has been observed in starved wild-type cells (mainly expressing sGC) that addition of GTP before GTPγS inhibits the stimulating effect of GTPγS (28). These observations suggest that the target of GTPγS rapidly hydrolyses GTP and slowly releases the produced GDP. These properties have often been found in small GTP-binding proteins that, unlike Gα, do not require a Gβ for activation. This may suggest that the guanylyl cyclases in *Dictyostelium* are regulated via the concerted activation of surface receptors, heterotrimeric G-proteins and small G-proteins. A similar hypothesis was recently obtained for *Saccharomyces cerevisiae* where Gβγ recruits Far1p leading to the activation of Cdc42p (38), as well as for other systems like fibroblasts, COS-7 cells, and human airway smooth muscle cells where G_i mediates the activation of $p21^{ras}$ (39–41).

The function of cGMP during chemotaxis and multicellular development is emerging. Previous mutant analysis uncovered several mutants with chemotaxis defects that have an altered cGMP metabolism (see Ref. 10), notably mutant KI-8, which has very low cGMP levels. In comparison with the absolute chemotaxis-null phenotype of KI-8, chemotaxis of gca^-/sgc^- double null cells is relatively normal. It has been demonstrated that cGMP induces the phosphorylation and rearrangement of myosin heavy chain II filaments. Chemotaxis of $mhcII^-$ null cells is disturbed but not absent. The preliminary phenotypic experiments on gca^-/sgc^- double null cells suggest that cGMP may play a role in chemotaxis through myosin filament formation but is not absolutely requires for chemotaxis as is the case for myosin heavy chain. Detailed computer-assisted chemotaxis analysis and phosphorylation of the myosin heavy and light chains of gca^-/sgc^- single and double null cells is in progress and should uncover the mechanism by which cGMP regulates chemotaxis.

The identification of two guanylyl cyclases in *Dictyostelium,* GCA and sGC, uncovers unexpected evolutionary traits. GCA has the topology of a 12-transmembrane adenylyl cyclase, whereas sGC is the homolog of a soluble adenylyl cyclase. No close phylogenetic relationship can be found with mammalian guanylyl cyclase (17, 18), suggesting that the mammalian guanylyl cyclases, GCA and sGC, each developed independently into a guanylyl cyclase. From a biochemical point of view this may not be very surprising, considering the relative ease by which an AC can be converted experimentally to a GC (31–34, 42, 43). However, from an evolutionary perspective, one would expect that AC to GC interconversions would have occurred more frequently. In the family of phosphodiesterases that hydrolyze cAMP and cGMP, a change of substrate specificity during evolution may have been relatively common (44, 45), but it seems also scarce in cAMP- and cGMP-dependent protein kinases. Apparently, the specificity of cyclases and kinases cannot be changed easily because the mechanisms that regulate cyclases and the substrate specificity of kinases have to remain functionally coupled.

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